Serial No. 10/038,984
Group No. 1635
Confirmation No. 9705

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/11, A61K 31/70, C12N 15/86

A1

(11) International Publication Number: WO 95/27783

(43) International Publication Date: 19 October 1995 (19.10.95)

(21) International Application Number: PCT/CA95/00190

(22) International Filing Date: 5 April 1995 (05.04.95)

(30) Priority Data:
08/223,560 6 April 1994 (06.04.94) US

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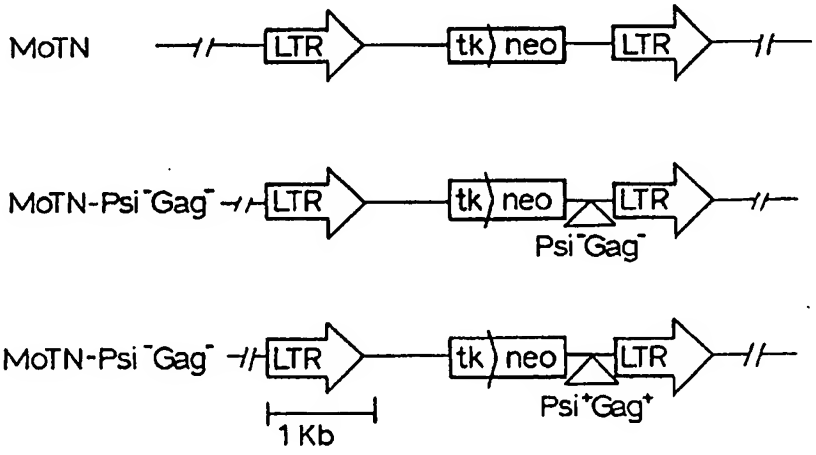
(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: INHIBITION OF HIV-1 MULTIPLICATION IN MAMMALIAN CELLS



(57) Abstract

A method of inhibiting human immunodeficiency virus type 1 (HIV-1) in a mammal using mammalian cells, particularly, human CD4 containing lymphocytes, which express chimeric RNA molecules containing HIV-1 ψ signal and/or Gag coding sequences in antisense orientation. HIV-1 production was delayed up to 30 days when compared with control cells lacking the test DNA sequences. Retroviral vectors expressing the chimeric RNA molecules are provided.

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INHIBITION OF HIV-I MULTIPLICATION IN MAMMALIAN CELLS

FIELD OF INVENTION

This invention relates to human immunodeficiency virus type 1 (HIV-1) and to inhibition of multiplication thereof in mammalian cells expressing chimeric RNA molecules containing HIV-1 packaging ψ signal and Gag coding sequences in antisense orientation; to said cells and therapeutic compositions comprising said cells; and retroviral vectors expressing said chimeric RNA molecules.

BACKGROUND TO THE INVENTION

Acquired immunodeficiency syndrome (AIDS) is caused by a retrovirus, called HIV-1, which mainly infects T-lymphocytes and monocytes/macrophages derived from haematopoietic stem cells.

An ideal step at which to inhibit virus multiplication would be during an early stage in the virus life cycle. However, if later stages during the virus life cycle are blocked, virus production will be inhibited, which in turn will prevent new rounds of infection. In this case, resistance will be confined to the second and subsequent rounds of infection. During the HIV-1 life cycle, Tat-TAR, Rev-RRE, and gag/ψ signal interactions are crucial for *trans*-activation, late gene expression, and virion RNA packaging, respectively. Interference during these processes may take place by providing the cell with interfering RNA or protein molecule(s); *e.g.* the sense (decoys of viral protein binding sites) or antisense RNAs to TAR, RRE, ψ signal, and Tat, Rev, or Gag open reading frames or trans-dominant mutants of HIV-1 Tat, Rev, or Gag proteins.

The Tat protein of HIV-1 allows trans-activation of HIV-1 gene expression, while Rev protein of HIV-1 allows the switch from early to late gene expression. While both of these genes overlap with each other, they are translated in different reading frames. Antisense RNA to HIV-1 Tat/Rev mRNA has been shown to confer resistance to HIV-1 infection in mammalian cell lines (1, 2, 3, 4).

Antisense RNA, complementary to a specific portion of HIV-1 RNA molecule, upon hybridization with target RNA sequences, disrupt reverse transcription, processing, translation, and/or transport of this RNA. Antisense RNAs have been shown to alter specific gene expression in several cell systems, including bacteria, Xenopus oocytes, Drosophila embryos, plants, and mammalian cells (5, 6). The degree of inhibition obtained in these studies was variable and depended upon many factors, including size, hybridization location, secondary structure, and level of expression of both the antisense RNA and the target mRNA whose expression was being modulated. Synthetic oligodeoxynucleotides, when added to the culture medium, have also been shown to inhibit HIV-1 multiplication.

A sense RNA approach has been used to block replication of the genome of a plant RNA virus by employing the origin of replication located at the 3' end of the genome as a competitive inhibitor for viral replicase (7). RNA-RNA and RNA-protein interactions are crucial for HIV-1 replication, trans-activation, transcription, transport, translation, and packaging, and the HIV-1 RNA sequences involved in these interactions are known. Non-HIV-1 RNAs containing TAR sequence in a sense orientation have been shown to compete with HIV-1 mRNAs for binding to RNA and/or protein and to result in inhibition of HIV-1 multiplication.

The *cis*-acting TAR element is a 59 nucleotide-long RNA stem-loop structure present at the 5' end of all HIV-1 transcripts (8). The Tat protein binds to a bulge region present within this structure. However, Tat binding in itself is not sufficient (9) and a number of specific TAR RNA-binding cellular proteins are required for HIV-1 *trans*-activation. Retroviral vectors expressing HIV-1 TAR RNA decoys (10, 11, 12, 13) have been shown to confer HIV-1 resistance. Retroviral vectors expressing antisense RNA to the HIV-1 *tat* gene have also been shown to confer HIV-1 resistance (1, 2, 3, 4, 14). However *trans*-dominant mutant of HIV-1 Tat protein (15), when expressed from retroviral vectors in either Tat- or Tat- and Rev-inducible manner, failed to protect cells against HIV-1 infection (16, 17). Vectors expressing antisense RNA targeted to the Gag mRNA 5' leader region (18, 19) have also been shown to inhibit HIV-1 multiplication.

The Gag (p55) and Gag-Pol (p160) polyproteins are translated from the 9.4 kilobase (kb) genomic mRNA. The *pol* gene is expressed as a result of frameshift

near the end of the *gag* reading frame. Following budding of virus particles, the viral protease cleaves the Gag polyprotein (p55) into the p17, p24, p7, and p9 proteins, and the Gag-Pol polyprotein (p160) into the p6, p11 (protease), p51, p64 (2 subunits of reverse transcriptase, RT), p15 (RNase H), and p34 (integrase) proteins.

RRE is a 234 nucleotide-long RNA sequence located within the *env* reading frame (20, 21). RRE has been predicted to form a highly complex secondary structure containing a central stem I surrounded by stem-loops II, III, IV and V (21). The 66 nucleotide-long stem-loop II has been found to contain the primary Rev binding site and is also sufficient for Rev response *in vivo* (22, 23). In the absence of Rev, the translation of unspliced and singly spliced mRNA into protein is prevented by *cis*-acting repressor sequences (CRS) present in the HIV-1 *gag*, *pol*, and *env* open reading frames (20, 24, 25). Rev-RRE interaction is sufficient to override the inhibitory action of the CRS such that these mRNAs can now reach the cytoplasm and become translated. Plasmids expressing one, three, and six copies of RRE have been shown to interfere with the HIV-1 Rev protein activity in a transient co-transfection experiment performed in HeLa cells (26); over expression of RRE decoys has also been shown to inhibit HIV-1 multiplication in CEM cells (27). As well, retroviral vectors allowing constitutive or Tat-inducible expression of *trans*-dominant mutants of either Rev (16) or Tat and Rev (15, 17, 28) were shown to confer resistance to HIV-1 infection.

The HIV-1 ψ signal is required in *cis* for specific recognition and packaging of the viral genomic RNA; two copies of the HIV-1 genomic RNA are encapsidated per virus particle. Nucleotides located between the major splice donor site and the Gag initiation codon are essential for HIV-1 RNA packaging (29, 30, 31); this region has been shown to fold into a stable secondary structure involving four stem-loops (32). The precise length of the HIV-1 ψ signal required for packaging is not known but it can be inferred from studies performed using Moloney murine leukemia virus (MoMuLV) (33) that it would be contained within 1000 nucleotides downstream of the primer binding site. The HIV-1 ψ signal is recognized *in cis* by the zinc finger motif within the nucleocapsid domain and by one other domain of the HIV-1 Gag polyprotein precursor (34, 35). Cells allowing constitutive expression of *trans* dominant mutant Gag proteins have been shown to repress HIV-1 replication (36).

Vectors expressing antisense RNAs targeted to the Gag mRNA 5' leader

region (18, 19) have been shown to inhibit HIV-1 multiplication.

REFERENCE LIST

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It would be of much value to provide retroviral vectors expressing anti-HIV-1 gene(s) to infect patients' bone marrow (BM) stem cells or peripheral blood lympocytes (PBLs) which, upon transplantation and differentiation, would potentially give rise to an HIV-1 resistant immune system.

As a result of extensive investigations, I have discovered methods of inhibiting the multiplication of HIV-1 in a mammalian cell.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method of inhibiting HIV-1 multiplication in a mammalian cell.

It is a further object of the present invention to provide cells of use in said method.

It is a yet further object of the present invention to provide therapeutic compositions comprising said cells.

It is a still yet further object of the present invention to provide vectors, particularly, retroviral vectors of use in the preparation of said cells.

Accordingly, in one aspect the invention provides a gene therapeutic method of inhibiting HIV-1 multiplication in a mammal comprising treating said mammal with an effective amount of mammalian cells expressing RNA molecules containing HIV-1 signal and/or Gag coding sequences in antisense orientation.

Preferably, the mammalian cells are human bone-marrow cells, and more preferably, blood cells.

In a further aspect the invention provides mammalian cells harboring proviral vector DNA expressing RNA molecules containing HIV-1 signal and/or Gag coding sequences in antisense orientation.

In a yet further aspect the invention provides a retroviral vector expressing RNA molecules containing HIV-1 signal and/or Gag coding sequences in antisense orientation.

Preferably, the retroviral vector is derived from the Moloney murine leukemia virus (MoMuLV).

Thus, in the development of the present invention, MoMuLV-derived retroviral vectors were engineered to express HIV-1 ψ signal and Gag coding sequences in anti-sense orientation in chimeric RNAs. These sequences were expressed under control of the herpes simplex virus (HSV) thymidine kinase (*tk*) promoter. Both, ψ signal and Gag coding sequences were expressed as part of the 3' untranslated region of the neomycin phosphotransferase (*neo*) mRNA. The constructs were used to transfect/infect packaging cell lines and the retroviral vector particles released were used to infect a human CD_4^+ lymphocyte-derived MT4 cell line. The stable MT4 transformants harbouring proviral vector DNA expressing ψ signal and Gag coding sequences in antisense orientation, were each tested for their susceptibility to HIV-1 infection. The stable MT4 transformants were then tested for interfering RNA (containing ψ signal and Gag coding sequences) production by reverse transcription-polymerase chain reaction (RT-PCR) analysis as well as for their susceptibility to HIV-1 infection. Compared to the results obtained with the control cells lacking any of the

test DNA sequence, the rate of HIV-1 production was delayed by up to 30 days in the antisense RNA-expressing cells. These results indicate that retroviral vectors expressing the HIV-1 ψ signal and Gag coding sequences in antisense orientation can be used to confer HIV-1 resistance.

In a further aspect, the invention provides a therapeutic composition for confering HIV-1 resistance to a mammal comprising cells as hereinbefore defined in association with a pharmaceutically acceptable carrier, diluent or adjuvant therefor. It will be readily understood by the person skilled in the art that the cells should be present in an effective therapeutic amount.

In gene therapy in patients using human peripheral blood lymphocytes (PBLs) and bone marrow (BM) cells, the source of $CD4^+$ lymphocytes and macrophages - major targets of HIV-1 infection - are the peripheral blood or bone marrow cells. PBLs are easy to access. However, these fully differentiated cells have a limited life span and therefore will only provide short term resistance. PBL gene therapy will therefore have to be repeated after a certain interval. BM cells contain stem cells which are capable of both self-renewal and differentiation into lymphocytes, macrophages, and other hematopoietic cells such as erythrocytes, granulocytes, and megakaryocytes. For gene therapy to have a sustained effect, it should be performed at the level of such self-renewing, pluripotent haematopoietic stem cells allowing continued production of progeny cells containing the therapeutic gene. As stem cells represent only 1 per 10,000 nucleated cells present within the bone marrow, a large number of bone marrow cells are infected by retroviral vectors to ensure transformation of these rare stem cells.

Gene therapy for the treatment of AIDS provided herein consists of using retroviral vectors to deliver the anti-HIV-1 RNA molecules to human PBLs and BM stem cells. Transformants selected in vitro are transplanted back to the patient. Following differentiation, these transformed cells lead to the development of an immune system in which various blood cells (including CD4 lymphocytes and macrophages) express the anti-HIV-1 RNA molecules and are therefore resistant to HIV-1.

Preferred methods of in vitro stimulation and culture for gene transfer into mammalian cells, particularly stem cells, with a gene transfer vector, particularly, a retrovious vector are disclosed in Canadian Patent Application No. 2086844,

published July 8, 1994 - Dube et al, which disclosure is included herein by reference.

Following appropriate stimulation and culture in vitro, PBLs and BM cells are infected by cocultivation with packaging cell lines producing retroviral vector particles. Transformants are then selected in vitro for growth in medium containing appropriate cytokines and antibiotics. These transformants are then transplanted back to the patient. Following transplantation and differentiation, blood samples are tested for anti-HIV-1 gene expression and for the ability of these cells to resist HIV-1 infection. Other disease symptoms, viral load and emergence of resistant HIV-1 isolates are examined as well.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the invention may be better understood, a preferred embodiment will now be described by way of example only, with reference to the accompanying drawings, wherein:

Fig.1 represents LTR-LTR sequences present in the proviral DNA integrated in the target cell line as part of the map of retroviral vectors expressing antisense RNA to HIV-1 ψ signal and Gag coding sequence; and

Fig. 2 shows the results of HIV-1 infections on a pool of stable MT4 transformants expressing HIV-1 ψ signal and Gag coding sequence-containing RNAs.

MATERIALS AND METHODS

Materials

All restriction enzymes were purchased from GIBCO BRL Ontario, Canada. T4 DNA ligase was obtained from Pharmacia, Quebec, Canada, and Calf intestinal phosphatase was obtained from Boehringer Mannheim, Quebec, Canada. Fetal bovine serum (FBS) was obtained from Hyclone and GIBCO BRL. Geneticin (G418), a mixture of antibiotic-antimycotic agents (containing penicillin, streptomycin, and Fungizone^R), L-Gln, Eagle's minimal essential medium, α-modification (α-MEM), and RPMI 1640 medium were purchased from GIBCO BRL.

Plasmid Construction

Unless otherwise stated, all recombinant DNA techniques were performed as described in (37, 38). The retroviral vector, pUCMoTN (39), used in this study, is derived from MoMuLV. This vector allow *neo* gene expression (conferring $G418^R$) under control of HSV *tk*. The ψ signal and Gag sequences were cloned in this vector as part of the 3' untranslated region of the *neo* mRNA.

The pUCMoTN-ψ^-Gag$^-$ and pUCMoTN-ψ^+Gag$^+$ vectors were constructed as follows.

A 4.0 EcoR1 fragment from pBKBHIOS (NIH#182) was cloned into the EcoRI site of the pUC18 vector (Pharmacia). A 1440 bp *Bam*HI-*Bgl*II fragment containing HIV-1 ψ signal and Gag coding sequences was isolated from this pUC-ψ vector. This fragment (containing *Sst*I-*Bgl*II sequences of HIV-1 strain HXB2) was cloned into the unique *Bam*HI site in the pUCMoTN-Rz1 vector (40). The resulting clones were characterized by restriction enzyme analysis and clones containing a single copy of the ψ signal and Gag coding sequences in antisense (pUCMoTN-ψ^-Gag$^-$) and sense (pUCMoTN-ψ^-Gag$^-$) orientations, with respect to the vector were selected.

Mammalian Cell lines

The ecotropic Psi-2 (41) and amphotropic PA317 (ATCC cat# CRL0978) (42) packaging cell lines were cultured in α-MEM medium supplemented with 2mM L-Gln, 0.1 volume of antibiotics/antimycotic solution (penicillin, 1000 units/ml; streptomycin, 1000 μg/ml; Fungizone^R, 2.5 μg/ml), and 10% FBS (Hyclone) at 37°C in a humidified atmosphere with 5% CO_2. The human CD_4^+ lymphocyte-derived MT4 suspension cell line, NIH Cat #120, was cultured in RPMI 1640 medium also supplemented with Gln, antibiotics/antimicotic agents, and FBS (GIBCO) as above and were incubated at 37°C in a humidified atmosphere with 5% CO_2. The selective media was prepared as above except that it also contained G418 (200 μg/ml for Psi-2 and PA317 cell lines; and 400 μg/ml for MT4 cell line).

Transfection and Infection of Mammalian Cell Lines

Psi-2 cells were transfected as follows using the Calcium phosphate co-precipitation technique (using the CellPhect Transfection Kit from Pharmacia): a 120

μl retroviral DNA solution (3 μg) was mixed with 120 μl Buffer A (0.5 M $CaCl_2$, 0.1 M HEPES) and incubated at 22°C for 10 min. An equal volume (240 μl) of Buffer B (0.28 M NaCl, 0.05 M HEPES, 0.75 mM NaH_2PO_4, 0.75 mM Na_2HPO_4) was added, mixed immediately by vortexing and incubated for 15 min. The mixture was then added drop wise to the cell culture (50% confluent in 60 mm plates containing 3 ml fresh medium). The cells were incubated under normal growth conditions for 6 hrs, then washed twice with fresh medium. The cells were subjected to glycerol shock with 1.5 ml 15% glycerol in 10 mM HEPES pH 7.5, 150 mM NaCl for 3 min at 22°C, then washed once with fresh medium. Fresh medium (5 ml) was added and the cells were grown under normal conditions for 2 days. On day 3, the transfected cells were washed once with phosphate-buffered saline (PBS) containing antibiotics/antimycotic agents, trypsinized with 0.05% trypsin, 0.53 mM EDTA-4Na (GIBCO), transferred to 100 mm plates and grown in selective medium containing 200 μg/ml G418. The medium was changed every 3-4 days until selection was complete (15-20 days). The number of resistant colonies was then determined. The cells were washed with PBS, trypsinized and re-seeded.

Vector particles released from the transformed Psi-2 cells were obtained by filtering culture medium from cells at 50-100% confluency through a 0.22 μm filter. These particles were used to infect PA317 cells as described previously (43). Essentially 2 x 10^5 cells were seeded for 6 hours in 60 mm tissue culture dishes in 4 ml medium, after which this medium was replaced by 1 ml medium containing 8 μg/ml polybrene and 100 μl vector particles. After a 2 hour incubation at 37°C, 3 ml medium was added and the incubation continued for 16 more hours. Cells were then trypsinized and transferred to 100 mm tissue culture dishes in the presence of selective medium containing 200 μg/ml G418. The selective medium was changed every 4-5 days and the number of colonies counted after 14 days. Vector particles released from the PA317 cells (50-100% confluent) were then collected and used to infect MT_4 cells.

The MT4 cells (3 x 10^5) were pelleted and resuspended in 0.5 ml RPMI 1640 medium containing 16 μg/ml polybrene. Vector particles (0.2 ml) and RPMI 1640 medium (0.3 ml) were then added and gently mixed to the cells. Cells were transferred to 60 mm petri dishes, and incubated under normal growth conditions for 2 hrs. Four ml of fresh RPMI 1640 medium were then added and the cells were grown overnight.

The infected cells were then centrifuged, resuspended in selective medium containing 400 μg/ml G418 and transferred to 100 mm petri dishes. Every 3-4 day, half of the cell suspension was removed and replaced with fresh selective medium. By day 20, all of the uninfected cells had died and the remaining stably transformed cells were frozen and were used in the following experiments.

Polymerase chain reaction (PCR) and reverse transcription (RT)-PCR analysis

Genomic DNA isolated (37) from the MT4 cells stably transformed with MoTN-ψ^- Gag^- vector particles was used in PCR as follows. PCR reaction (100 μl) was performed in the presence of $MgCl_2$ (1.5 mM), oligonucleotides (20 mM each; amplification buffer (1 x concentration; Promega), dNTPs (10 mM each), genomic DNA (1 μg), and Taq polymerase (2 units, Promega). The samples were overlaid with 100 μl mineral oil and amplified using Perkin-Elmer Cetus Instruments DNA Thermal Cycler by using three linked files as follows: File 1, STEP-CYCLE 1 min at 95 °C; File 2, STEP-CYCLE 1 min at 55 °C; File 3, STEP-CYCLE 1 min at 72°C; with a total of 45 cycles. PCR products (10 μl aliquots) were then analyzed by electrophoresis on a 3% agarose gel.

In order to assess the amount of ψ^- Gag^- RNA expressed, RT-PCR was performed using RNA isolated from the MT4 cells stably transformed with MoTN-ψ^- Gag^- vector particles. The cells were grown for 48 hrs and then the total RNA was extracted using the Guanidium thiocyanate-Phenol-Chloroform procedure (44). Reverse transcription was performed as follows: total RNA (5 μg) was incubated with oligo dT (20 mM) for 10 min at 65°C in a total volume of 20 μl. The reaction mixture was chilled on ice for 2 min and RNA guard (75 units; Pharmacia), reverse transcription buffer (1 x concentration; BRL), DTT (5 mM), dNTPs (12.5 mM each), and Superscript RTase (400 units; BRL) were added to make a total volume of 40 μl. The reaction mixture was incubated for 1 h at 37°C and then for 10 min at 65°C. Five μl of this reaction mixture containing cDNA was then used in a PCR reaction which was performed as described above and the products were then analyzed by electrophoresis on a 3% agarose gel.

HIV-1 infections

Actively dividing various MT4 transformants (1 x 10^6 cells/ml) were each infected with HIV-1 as follows: a 2 ml cell culture was incubated with 20 μl HIV-1 strain NL4-3, NIH Cat #78, ($10^{6.4}$ $TCID_{50}$/ml) for 2 hrs at 37°C. The cells were then pelleted, washed 3 times with PBS, resuspended in 2 ml medium, transferred to 35 mm dishes and allowed to grow at 37°C. Every 3 days for up to day 30, a 1 ml sample containing cells and medium from each infected cell culture was removed and frozen at -70°C. One ml of complete medium was added back to the culture each time. After day 30, the frozen samples were thawed and centrifuged at 250 x g for 10 min. The culture supernatants (200 μl each) were then diluted as required and tested for the presence of HIV-1 p24 antigen using the HIVAG-1 Enzyme Immunoassay Kit (Abbott Laboratories) according to the manufacturer's instructions. A standard curve (OD_{492} as a function of p24 antigen concentration in ng/ml) was obtained using p24 antigen provided by the supplier. The OD_{492} values for the various samples collected at different time intervals following HIV-1 infection were corrected for the dilution factor and were converted to ng p24 antigen released per ml cell culture supernatant. These experiments were repeated four times.

RESULTS

Retroviral vectors allowing interfering RNA expression

MoMuLV-derived retroviral vector pUCMoTN was modified to express HIV-1 ψ signal and Gag coding sequences in antisense orientation. This molecule was expressed as part of the 3' untranslated region of the *neo* mRNA in between the stop codon and the poly(A) site (Fig. 1).

The pUCMoTN-ψ^- Gag^- vector expressed a single copy of antisense RNA to both of the HIV-1 ψ signal and Gag coding region (Fig. 1). The expression of HIV-1 ψ signal and Gag coding region-containing RNAs in these vectors is under the control of the MoMuLV 5' LTR and HSV *tk* promoters.

Establishment of stable MT4 transformants expressing interfering RNA molecules

The aforementioned retroviral vectors were first used to transfect an ecotropic packaging cell line Psi-2; (41), and the vector particles released from this cell line were

used to infect an amphotropic packaging cell line (PA317; 42). The resulting amphotropic pseudotyped retroviral vector particles capable of infecting human cells were then used to infect a human $CD4^+$ lymphoid (MT4) cell line and the $G418^R$ stable transformants were selected. The presence of the anti-HIV-1 gene and the level of therapeutic RNA/protein produced in these cells were then monitored as described below.

Confirming CD_4 expression on stably transformed MT4 cells

The fact that various MT4 transformants expressed CD4 was confirmed by Fluorescence activated cell sorter (FACS) analysis using an anti-CD4 monoclonal antibody (T4-RD1/T11-FITC). Over 95% of cells examined were found to be CD4 positive.

Confirming the presence of interfering RNA

Since various interfering RNA molecules are expressed as part of the *neo* gene, the fact that the cells are $G418^R$ confirms that the *neo* mRNA was expressed.

Anti-HIV-1 resistance was obtained from MT_4 cells stably transformed with the MoTN-ψ^- Gag^- vector particle. The presence of HIV-1 ψ signal and Gag coding sequences within their genome was confirmed by PCR analysis. The presence of ψ signal and Gag coding sequence-containing RNAs was confirmed by RT-PCR. As expected, a 315 bp PCR or RT-PCR product was visible in both cases.

Testing for the ability of interfering RNAs to confer HIV-1 resistance

MT4 cells stably transformed with MoTN-ψ^- Gag^- vector particles expressing antisense RNA to HIV-1 ψ signal and Gag coding sequences were challenged with HIV-1. MT4 cells transformed with the parental retroviral vectors lacking test DNA sequences served as control. Virus production was monitored by measuring the level of p24 antigen (HIV-1 *gag* gene product) in the cell culture supernatant every 3 days for up to 30 days post-infection. The MT4 transformants expressing antisense RNA to the ψ signal and Gag coding sequences delayed virus production for up to 30 days (Fig. 2).

HIV-1 resistance of sense RNA-expressing cells was monitored as

follows. MT4 cells stably transformed with MoTN-ψ^+ Gag^+ vector particles were subjected to challenge by HIV-1 and virus production in the culture supernatant was measured every 3 days post-infection. MT4 cells expressing sense RNA to the HIV-1 ψ sequence and Gag coding sequences failed to prevent HIV-1 multiplication (Fig. 2).

In the studies of the present invention, retroviral vectors were, thus, engineered that expressed ψ signal and Gag coding sequences in antisense orientation. The retroviral vector particles were used to infect the human CD_4^+ lymphocyte-derived MT4 cells and stable $G418^R$ transformants were selected. The pool of these transformants was then infected with HIV-1 and virus production measured for up to 30 days post-infection.

Interference with genomic RNA packaging is believed to result in the production of replication-defective virions, thus limiting the spread of viral infection. In cells expressing HIV-1 ψ signal and Gag coding sequences in sense orientation, HIV-1 production began even earlier than in the control cells (Fig. 2). If the HIV-1 ψ signal and Gag coding sequence-containing retroviral vector RNA was also packaged by HIV-1, the infectivity of these chimeric RNA-containing virus particles should have been reduced. The lack of resistance observed with the HIV-1 ψ signal and Gag coding sequences expressed in the sense orientation may be explained by the fact that the length of the HIV-1 ψ signal used in the present experiments was not sufficient to allow packaging of non-viral mRNA.

High level of resistance was observed in cells expressing HIV-1 ψ signal and Gag coding sequences in an antisense orientation as no virus could be detected in the culture supernatants from these cells for up to 30 days post-infection (Fig. 2). Note that for the antisense RNA approach to be effective, it is not required that the antisense RNA to the HIV-1 ψ signal be designed against the entire HIV-1 ψ signal. However, when MT_4 transformants were challenged with a higher dose of HIV-1, some HIV-1 production could be detected around day 20 but it remained quite low; by day 30, the amount of virus produced was 6-7 fold below the control. As expected, these cells were fully viable.

The experiments described hereinabove were performed using a pool of transformed MT_4 cells and therefore represent an average of the resistance conferred by each cloned MT_4 transformant. This appears to be a more realistic view of what

would happen should human gene therapy be performed using these retroviral vectors.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention as described and claimed.

I claim:

1. A method of inhibiting HIV-1 multiplication in a mammal comprising treating said mammal with an effective amount of mammalian cells expressing RNA molecules containing HIV-1 ψ signal and/or Gag coding sequences in antisense orientation.
2. A method as claimed in Claim 1 wherein said mammal is a human and said cells are bone-marrow cells.
3. A method as claimed in Claim 1 wherein said cells are blood cells.
4. A method as claimed in Claim 1 wherein said cells are human CD4 containing lymphocytes.
5. A method as claimed in Claim 1 wherein said cells express RNA molecules containing HIV-1 packaging signal coding sequence in antisense orientation.
6. A method as claimed in Claim 1 wherein said cells express RNA molecules containing HIV-1 Gag coding sequence in antisense orientation.
7. Mammalian cells harboring DNA expressing RNA molecules containing HIV-1 ψ signal and Gag coding sequences in antisense orientation.
8. Mammalian cells as claimed in Claim 7 harboring proviral vector DNA expressing RNA molecules containing HIV-1 ψ signal and/or Gag coding sequences in antisense orientation.
9. Cells as claimed in Claim 7 being bone-marrow cells.
10. Cells as claimed in Claim 7 being blood cells.
11. Cells as claimed in Claim 7 being human CD4 containing lymphocyte cells.
12. A therapeutic composition comprising mammalian cells as defined in any one of Claims 7-11, harboring DNA expressing RNA molecules containing HIV-1 ψ signal and/or Gag coding sequences in antisense orientation; and a pharmaceutically acceptable diluent, adjuvant or carrier therefore.
13. A vector expressing RNA molecules containing HIV-1 ψ signal and/or gag coding sequences in antisense orientation.
14. A vector as claimed in Claim 13 expressing RNA molecules containing HIV-1 ψ signal coding sequence in antisense orientation.
15. A vector as calimed in Claim 13 expressing RNA molecules containing HIV-1 gag coding sequence in antisense orientation.

16. A retroviral vector expressing RNA molecules containing HIV-1 ψ signal and/or Gag coding sequences in antisense orientation.

17. A retroviral vector as claimed in Claim 16 expressing RNA molecules containing HIV-1 ψ signal coding sequence in antisense orientation.

18. A retroviral vector as claimed in Claim 16 expressing RNA molecules containing Gag coding sequence in antisense orientation.

19. A retroviral vector as claimed in Claim 16 derived from Moloney murine leukemia virus (MoLuLV).

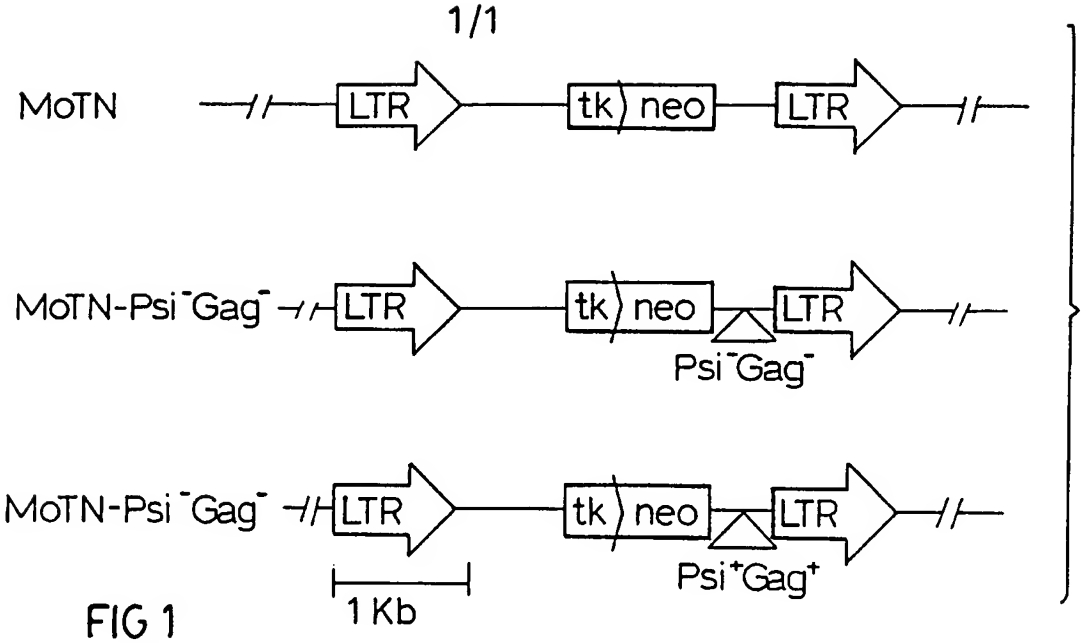
MoTN
LTR
tk neo
LTR
MoTN-Psi⁻Gag⁻
LTR
tk neo
LTR
Psi⁻Gag⁻
MoTN-Psi⁻Gag⁻
LTR
tk neo
LTR
Psi⁺Gag⁺
1 Kb

FIG 1

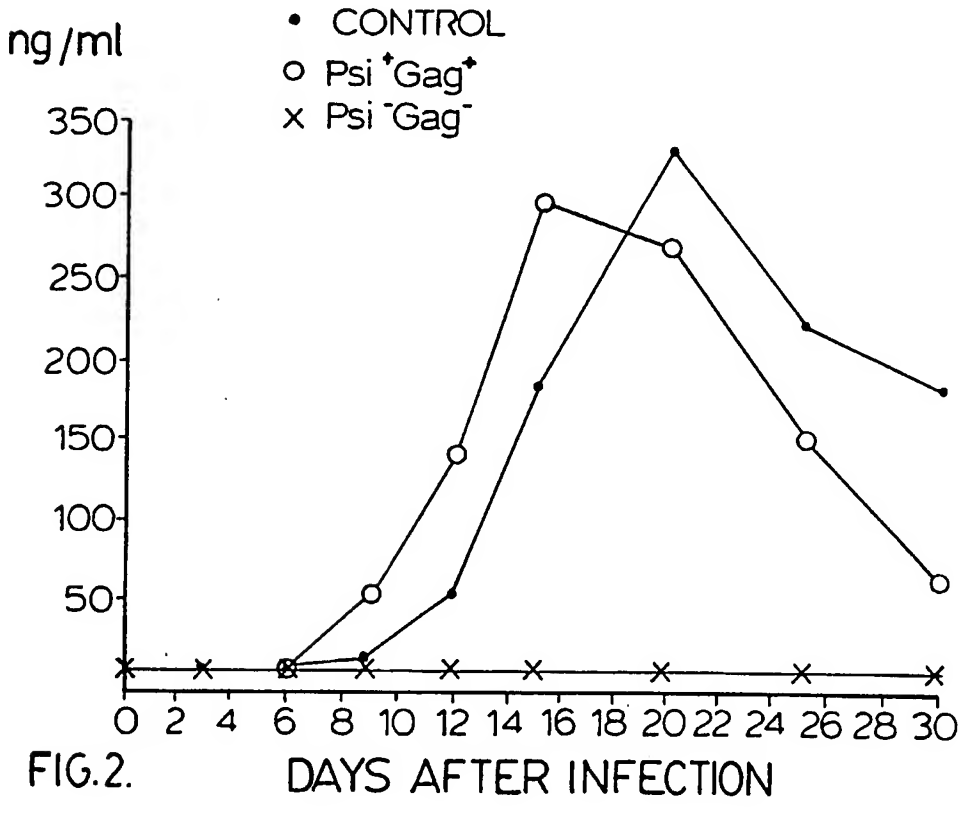
ng/ml
• CONTROL
o Psi⁺Gag⁺
x Psi⁻Gag⁻
350
300
250
200
150
100
50
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
DAYS AFTER INFECTION

FIG.2.

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/CA 95/00190

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 A61K31/70 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO-A-92 17211 (EDISON ANIMAL BIOTECHNOLOGY CE) 15 October 1992 see page 13 - page 15 see page 17 - page 20, line 22	1-5,13, 14,16, 17,19
Y	see page 25, line 20 - page 26, line 12 see page 30, line 24 - page 31, line 9 see examples --- -/--	2-4, 8-12, 16-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 June 1995	- 3. 07. 95

Name and mailing address of the ISA	Authonzed officer
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INTERNATIONAL SEARCH REPORT

Internat Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y		2-4, 8-12, 16-19

X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 169, no. 2, 15 June 1990 pages 643-651, XP 000128970 SCZAKIEL, G. ET AL. 'SPECIFIC INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REPLICATION BY RNA TRANSCRIBED IN SENSE AND ANTISENSE ORIENTATION FROM THE 5'-LEADER/GAG REGION' cited in the application see the whole document	1,3,4,6, 13,15

X	EP-A-0 331 939 (GREATBATCH GEN AID LTD) 13 September 1989 see examples 2,10 see claims	1,2,5, 13,14

X	NUCLEIC ACIDS RESEARCH, vol. 19, no. 7, 1991 OXFORD GB, pages 1421-1426, RITTNER, K. & SCZAKIEL, G. 'Identification and analysis of antisense RNA target regions of the human immunodeficiency virus type 1' see figure 1	1,3,4,6, 13,15

X	Wickstrom, E. (eds) 'Prospects for antisense nucleic acid therapy of cancer and AIDS'; 1991; Wiley-Liss, INC. pages 179-193; SCZAKIEL, G. et al.:' Human Immunodeficiency virus type-1 replication is reduced by intracellular antisense RNA expression' see the whole document	1,3,4,6, 13,15, 16,18

A	WO-A-94 01551 (US GOVERNMENT) 20 January 1994 see page 10, line 7 - line 18 see figure 1; examples	1,3-15

A	WO-A-90 13641 (SLOAN KETTERING INST CANCER) 15 November 1990 see page 16, line 27 - page 19, line 24	16-19

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	ANTISENSE RESEARCH AND DEVELOPMENT, vol. 4, 1994 US, pages 19-26, COHLI, H. ET AL. 'Inhibition of HIV-1 multiplication in a human CD4+ lymphocytic cell line expressing antisense and sense RNA molecuzles containing HIV-1 packaging signal and rev response element(s)' see the whole document ---	1-5,13, 14,16, 17,19
P,X	EP-A-0 612 844 (ORTHO PHARMA CORP) 31 August 1994 see column 2, line 50 - column 3 see column 8, line 13 - line 43 see figure 1 ---	1-8, 10-18
P,X	EP-A-0 598 935 (BAYER AG) 1 June 1994 see the whole document -----	1-4,6, 13,15

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Information on patent family members

Internat Application No
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		FI-A-	940867	26-08-94
		JP-A-	6335392	06-12-94
		NO-A-	940624	26-08-94
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